failing to teach the expression of Fas on liver cells in primary biliary cirrhosis.

Harada et al. is asserted to teach that Fas is expressed on biliary epithelial cells and that Fas antigen was found on the interlobular bile ducts of primary cirrhosis. The Examiner asserts that Harada et al. therefore teach that biliary epithelial cells in primary biliary cirrhosis undergo apoptosis in response to Fas/Fas ligand crosslinking, suggesting the involvement of apoptosis in the progression of bile duct injury and loss.

Shirakawa et al. is asserted to teach an antibody directed to Fas ligand and a method of treating systemic or pathological conditions caused by the Fas/Fas ligand interaction.

The Examiner asserts that the combined references teach the use of a Fas antagonist to prevent the Fas/Fas ligand interaction and that bile duct disappearance syndrome is cased by primary biliary cirrhosis, therefore the same mechanism is involved with both conditions. As such, it is asserted to be obvious to treat primary biliary cirrhosis by inhibiting the Fas/Fas ligand interaction.

Applicants traverse this rejection and withdrawal thereof is respectfully requested. The present invention, as most broadly encompassed by claim 8, is drawn to a method for preventing and treating hepatic cirrhosis or bile duct disappearance syndrome by administering a Fas antagonist to a patient.

As discussed above, the Examiner bases the rejection in part on the teaching in Harada et al. that Fas is expressed on biliary epithelial cells and that Fas antigen was found on the interlobular bile ducts of primary cirrhosis. At the crux of the rejection is the conclusion by the Examiner that this teaching in Harada et al. demonstrates that biliary epithelial cells in primary biliary cirrhosis undergo apoptosis in response to Fas/Fas ligand crosslinking, suggesting the involvement of apoptosis in the progression of bile duct injury and loss. However, as will be shown below, this conclusion is not supported by the reference.

As the Examiner notes, Harada et al. does teach that the interlobular bile ducts of primary biliary cirrhosis (PBC) frequently expressed CD95 (Fas) antigen in a cytoplasmic and membranous pattern, and that a high level of CD95 ligand (Fas-ligand) positive mononuclear cells were found in the same pathology samples. However, the Examiner's extrapolation of these results to the conclusion that Harada et al. demonstrate that biliary epithelial cells undergo apoptosis in response to Fas/Fas ligand crosslinking is unsupported and contrary to the accepted teachings in the field at the time of the invention.

Attached hereto is a journal article of Graham et al. Eur. J. Gastroenterology & Hepatology 110:553-557 (1998), wherein the investigators examined the expression of apoptosis related proteins in PBC and normal liver control tissue and found no change in

CD95/Fas or p53 expression. See Abstract; page 555, right column, lines 8-9 and page 556, left column, lines 11-10 from the bottom.

In addition, Graham et al. state on page 556, right column, lines 5-8,

> How these cells may be inducing apoptosis is unknown but a Fas-mediated mechanism is unlikely in view of the low level of expression we have seen.

Thus, the relationship between the pathology of PBC and apoptosis mediated by the Fas/Fas ligand pathway remains unknown, controversial and perceived differently depending on the investigator. In addition, even if Fas is expressed in a particular pathology, it is not possible to determine whether that expression results from or causes the disease. Even if Fas expression is demonstrated with a disease, that does not mean, nor is it possible to predict, that administration of a Fas antagonist will have any efficacy in treating the disease. Pharmacological testing with a clinical model is required before a prediction of efficacy can be made. Thus, even if the expression of Fas in PBC cells suggests that there is some relationship between PBC and Fas, it is not possible to predict what that relationship might be or whether a Fas antagonist will have an effect on the disease.

The field of the invention was highly unpredictable at the time the invention and it was not possible to predict or conclude from the prior art whether a Fas antagonist would be effective in treating PBC. The present inventors have demonstrated for the

first time that administration of a Fas antagonist is efficacious in treating PBC. This finding could not be predicted from nor is obvious over the prior art. As such, withdrawal of the rejection is respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact MaryAnne Armstrong, PhD. (Reg. No. 40,069) at the telephone number below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
Gerald M. Murphy, Jr., #28,977

MaryAnne Armstrong, PhD., #40,069

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

GMM/MAA/csm
1110-0279P

Attachment: Graham et al., Eur. J. Gastro. & Hep. (1998)

Bile duct cells in primary biliary cirrhosis are 'primed' for apoptosis

Alexandra M. Graham, Matthias M. Dollinger, Sarah E.M. Howie and David J. Harrison

Objective Primary biliary cirrhosis (PBC) is characterized by progressive, immune-mediated destruction of bile ducts (< 75 μm diameter) and secondary changes related to cholestasis which may involve apoptosis. In this study we sought to examine the protein expression of genes involved in apoptosis in biliary epithelium of PBC cases.

Design In order to investigate the susceptibility of biliary epithelial cells to apoptosis and their ability to proliferate, we examined the expression of a number of apoptosis related proteins in early and late stage PBC and histologically normal liver control tissue using immunohistochemistry.

Methods Liver biopsies from 15 early (stages I and II) and 14 late (stages III and IV) cases of PBC and 15 normal cases were examined immunohistochemically for expression of p53, CD95/Fas, bax, bcl-x, bcl-2 and the proliferation marker Ki-67.

Results CD95/Fas, bax and bcl-x were identified in biliary epithelium in 8/15, 11/15 and 8/15 normal biopsies. Weak expression of bcl-2 was found, but p53 was not identified. In cases of PBC surviving bile ducts showed strong bax and bcl-x expression. Inflammatory infiltrates were strongly bcl-2 positive. In cases showing a marked ductular reaction there was increased reactivity for bax and bcl-x in ductules. No change in CD95/Fas or p53 expression was seen. An increase in Ki-67 positive biliary epithelial cells was seen in PBC cases, indicating cell cycle activity.

Conclusions Bile duct epithelium constitutively expresses several genes involved in the execution of apoptosis but these cells also retain the ability to proliferate.

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Keywords: apoptosis, bax, bcl-2, Fas, primary biliary cirrhosis

University Department of Pathology, Edinburgh, UK

Correspondence to A.M. Graham, University Department of Pathology, Medical School, Teviot Place, Edinburgh, EH8 9AG, UK.
Tel: 0044 131 650 2960; Fax: 0044 131 650 6526;
e-mail: ag@srv4.med.ed.ac.uk

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Introduction

Primary biliary cirrhosis (PBC) is a chronic, slowly progressive cholestatic liver disease characterized by destruction of bile ducts of < 75 μm diameter [1]. The destruction is thought to be the result of apoptosis of biliary epithelial cells, triggered by autoimmune mechanisms [reviewed in References 2 and 3]. The aetiology remains unknown although various factors such as HLA type [4] and microbiological infection have been invoked [5–8]. One of the major diagnostic criteria for PBC is the presence of high titres of anti-mitochondrial antibodies in patients' sera, frequently specific for the E2-subunit of pyruvate dehydrogenase complex (PDC-E2) [reviewed in Reference 9]. However, the precise role of autoantibodies in pathogenesis has not been established.

$CD8^+$ T lymphocytes are present in inflammatory infiltrates within the livers of PBC patients [10–13]. They are centred around small bile ducts and may initiate damage directly through apoptosis induced via CD95/Fas and CD95L/Fas ligand or perforin mediated cytotoxic mechanisms [14]. Thus, cellular components of the immune response around the targeted bile ducts may release cytokines, generating an environment which increases the neighbouring biliary epithelial cells' sensitivity to apoptosis. Tumour necrosis factor alpha (TNFα) [15], interferon gamma (IFN-γ) [16] and transforming growth factor beta (TGFβ) [17–19] have been shown to cause apoptosis of hepatocytes and mRNA for each has been detected in human PBC liver [20–22].

The reason why biliary epithelial cells are specifically targeted in this disease and are vulnerable to death as an initiating event is unknown, but it does not appear to be the result of aberrant MHC Class II expression or antigen presentation by these cells as such changes occur late in the disease [23,24].

To test the hypothesis that in PBC the sensitivity of biliary epithelial cells to apoptosis is altered we examined the expression of a number of apoptosis related proteins in the bile ducts and liver of early and late stage PBC and normal control tissue. p53, Fas/CD95 and members of the bcl-2 family have been shown to interact with one another, for example p53 transcriptionally regulates Fas/CD95 and bax [25–27], and members of the bcl-2

family homo- and heterodimerize [reviewed in Reference 28]. The proliferative status of biliary epithelial cells was examined using the MIB-1 antibody against Ki-67 [29].

Methods

Biopsies

The diagnosis of PBC was made after full clinical, serological and histological assessment. Fifteen cases showed predominantly histological features seen in stage I or II (14 women, one man) and 14 showed stage III or IV (13 women, one man) according to the proposed model of disease progression reported by Scheuer [30]. Fifteen control liver samples were obtained from patients during routine lymphoma staging, psoriasis patients prior to commencing methotrexate therapy, or laparotomy during resection of colon cancer (six women, nine men). All liver tissue in the control group was reported as histologically normal and there was normal liver biochemical and synthetic function. Clinical details of the PBC cases are shown in Table 1.

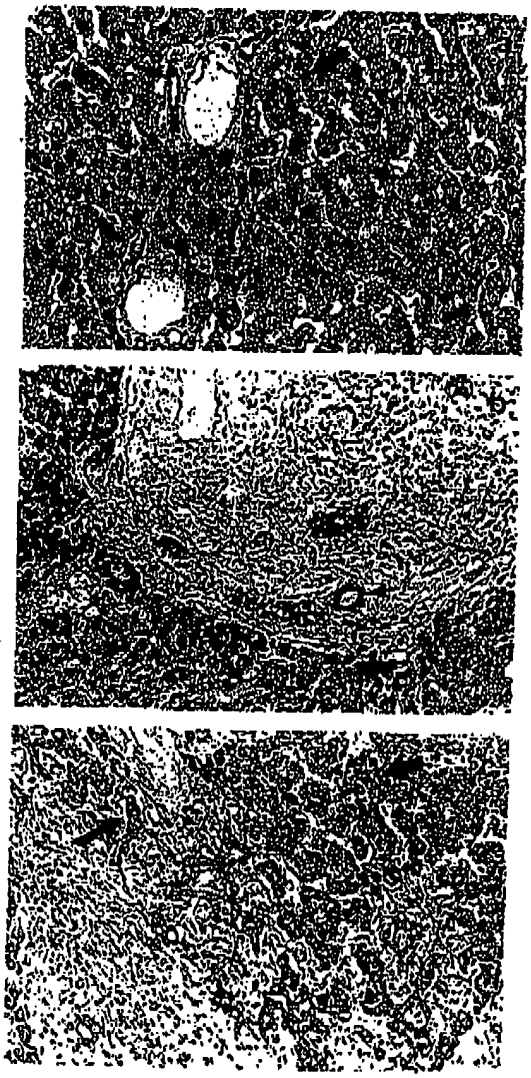
Immunohistochemistry protocols

Liver tissue was fixed in 10% buffered formalin and processed to paraffin. Sections of 3 μm thickness were cut onto Dako ChemMate Capillary Gap Microscope Slides (75 mm). Sections were dewaxed in xylene and rehydrated through descending alcohols. Pre-treatment with trypsin (ICN Biomedicals Inc.) (0.1% trypsin, 0.1% calcium chloride (Sigma), pH 7.8 at 37°C for 17 min) was required with the anti-bcl-x (Autogen Bioclear UK Ltd, dilution 1/100) and anti-bax (Autogen Bioclear UK Ltd, dilution 1/200) antibodies. Microwave antigen retrieval (2 × 5 min at 1000 W; slides immersed in 1.05 g citric acid (BDH Chemicals Ltd)/500 ml H_2O, pH 6.0) was required for the anti-bcl-2 (Dako UK, dilution 1/50), anti-CD95/Fas (Immunotech UK, dilution 1/200) and anti-Ki-67 (Immunotech UK, dilution 1/100) antibodies. p53 (Dako UK) was used at 1/100 dilution. All sections were washed in running tap water then phosphate buffered saline (Oxoid)/0.1% Tween 20 (Sigma) prior to loading onto a Dako Techmate 500 automated immunocytochemistry stainer according to the manufacturer's instructions using a streptavidin/biotin and horseradish peroxidase detection system with 3',3'-diaminobenzidine as chromogen. Negative controls omitting primary antibody were included.

Table 1 Clinical data assessing liver function of patients at time of liver biopsy. Median and (range) values of bilirubin, alanine aminotransferase, alkaline phosphatase and albumin of PBC patients at time of biopsy

Stage	Bilirubin	Alanine aminotransferase	Alkaline phosphatase	Albumin
Normal range	5–17 μM	< 35 U/l	< 130 U/l	40–50 g/l
Histological early disease (Stage I, II)	11 (5–60)	69.5 (22–194)	200 (104–861)	41.5 (29–48)
Histological late disease (Stage III, IV)	56.5 (13–256)	97 (20–845)	402.5 (116–1269)	32 (22–43)

Fig 1



(a) Expression of bax in normal bile ducts (→) and hepatocytes (↗). (b) PBC stage I liver showing strong expression in bile ducts (→) and upregulation of bax in hepatocytes (↗). (c) PBC stage II liver showing upregulation of bax in 'metaplastic ductules' (→) and hepatocytes (↗). Original magnification × 20.

Analysis of results

It was noted that the intensity of immunostaining for bcl-2, bax, bcl-x and Fas/CD95 was homogeneous with little variation between cells within a single duct or between ducts. For this reason biopsies were scored as follows by three independent observers: strong (that is

clearly visible granular stain at low power examination (× 4)); moderate (definite but weak immunopositivity visible at low power but needing confirmation by high power microscopic examination (× 25)); or weak/negative (equivocal staining, not consistently greater than in negative control section where primary antibody was omitted). Where inter-observer discrepancies were observed results were recorded after discussion of the individual case.

When Ki-67 or p53 were present they produced clear, discrete nuclear staining. Cases were scored positive if one or more nuclei of biliary epithelial cells was stained and negative if no positive cells were seen. Thereafter an estimate was made of the proportion of individual biliary epithelial cells stained.

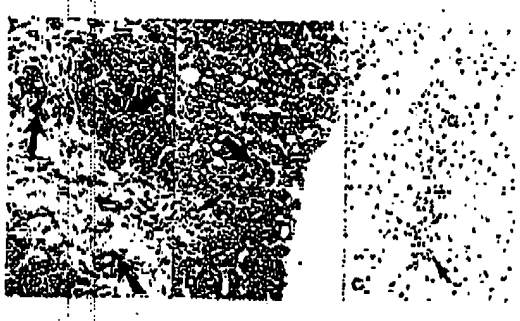
Bile ducts were classified as small (<75 μm) or large (>75 μm) by measurement using the HOME (Highly Optimized Microscope Environment) semi-automated computer system.

Results

Normal liver

bax (Fig. 1a) and bcl-x (Fig. 2b) were expressed uniformly in both hepatocytes and bile ducts but were not detected in Kupffer cells or endothelial cells. Moderate bax expression was seen in bile ducts < 75 μm in 11/15 sections (Table 2) and moderate bcl-x expression in 8/15 sections (Table 3). Only 3/15 cases expressed detectable bcl-2 in biliary epithelium. bcl-2 was not detected in hepatocytes. Eight of fifteen cases showed moderate staining of biliary epithelium with the anti-Fas/CD95 antibodies. In all cases hepatocytes were positive, the staining pattern being granular as previously reported in paraffin embedded tissue [31,32]. p53 was not detected and biliary epithelial cells did not express Ki-67.

Fig. 2



Expression of bcl-x in (a) PBC stage IV liver hepatocytes ( ) and 'metaplastic ductules' bile ducts ( ) showing upregulation, compared with (b) histologically normal liver showing only mild steatosis; hepatocytes ( ) and bile ducts ( ). (c) Negative control omitting primary antibody on histologically normal liver section; bile duct ( ). Original magnification × 20.

PBC liver

Residual bile ducts showed strong staining of bax (Table 2, Fig. 1b) and bcl-x (Table 3) which appeared more intense than in normal liver. bax and bcl-x were expressed strongly in 'metaplastic' biliary ductules (Figs 1c and 2a). Lymphocytes showed intense expression of bcl-2. bcl-2 expression seen in surviving biliary epithelial cells was weak and inconsistent, similar to normal liver. No change in Fas/CD95 staining was noted. p53 was not expressed. In PBC cases up to 8% of biliary epithelial cells in large ducts and in residual small ducts expressed Ki-67 (Fig. 3). The number of positive nuclei seen was greatest in ducts > 75 μm in sections showing early stage PBC. Areas of ductal 'metaplasia' remained Ki-67 negative.

Table 2 Results of bax immunocytochemistry in bile ducts < 75 μm and > 75 μm in normal and early and late stage PBC liver biopsy material. The figures are the number of cases with detectable strong, moderate and weak/negative expression. Expression when detected was homogeneous and uniform. *n*, total number of sections

	bax (< 75 μm)				bax (> 75 μm)			
Stage	Strong	Moderate	Weak/negative	*n*	Strong	Moderate	Weak/negative	*n*
Normal	0	11	4	15	0	10	2	12
Early	14	1	0	15	12	0	0	12
Late	6	4	4	14	8	3	3	14

Table 3 Results of bcl-x immunocytochemistry in in bile ducts < 75 μm and > 75 μm in normal and early and late stage PBC liver biopsy material. The figures are the number of cases with detectable strong, moderate and weak/negative expression. Expression when detected was homogeneous and uniform. *n*, total number of sections

	bcl-x (< 75 μm)				bcl-x (> 75 μm)			
Stage	Strong	Moderate	Weak/negative	*n*	Strong	Moderate	Weak/negative	*n*
Normal	0	8	7	15	0	8	4	12
Early	14	0	1	15	11	0	1	12
Late	13	1	0	14	13	1	0	14

Fig. 2

Proliferating biliary epithelial cell (⇑) and infiltrating cells (↗) expressing Ki-67 in PBC stage I liver. Original magnification × 20.

Discussion

We have shown that bile ducts constitutively express pro-apoptotic proteins, particularly bax, and the apoptotic regulator bcl-x. In contrast the anti-apoptotic protein bcl-2 is barely expressed and thus appears to play little part in regulating apoptosis in biliary epithelial cells in the cases we have studied. In addition to having a phenotype that allows apoptosis there is evidence that biliary cells retain the ability to proliferate, as shown by the positive Ki-67 staining seen in PBC liver. This supports the observation of Nakanuma and Hanada [33] who showed increased proliferative activity of epithelial cells in affected ducts. Charlotte *et al.* [34] have reported that normal bile ductules and small bile duct epithelium, but not large bile duct epithelium or hepatocytes, show weak expression of bcl-2 as detected immunohistochemically. However, Kuroki *et al.* [35] found no bcl-2 expression in biliary epithelium of normal or PBC livers, and other studies have shown more prominent bcl-2 staining in ductules at the borders of cirrhotic nodules relative to normal liver [36]. The absence of bcl-2 expression we report in hepatocytes is consistent with previous observations [34,37]. We have shown weak expression of Fas/CD95 in bile ducts supporting the findings of Kuroki *et al.* [35] and confirm its presence in hepatocytes [31,38–40]. The antiserum used against Fas/CD95 did not detect increased levels of Fas in the PBC livers. The presence of TNFα and IFNγ in PBC liver [20–22] might suggest an accompanying elevation in Fas expression. However, many of the experiments showing increased Fas expression by TNFα and IFNγ are *in vitro* studies using high doses of cytokine [41,42]. PBC is a chronic disease and levels of these cytokines present in the liver have not been quantified. Any changes that they may induce in Fas expression might not be sufficient for detection by immunohistochemistry, or in this disease these pleiotropic cytokines may be exerting other effects on the disease state independent of Fas.

Apoptosis of biliary epithelial cells in PBC has been shown [32,43,44] and this may be associated with the presence of cytotoxic $CD8^+$ T cells [10,11,13]. How these cells may be inducing apoptosis is unknown but a Fas-mediated mechanism is unlikely in view of the low level of expression we have seen. Such mechanisms have been invoked in a number of inflammatory diseases of the liver, especially viral hepatitis [31,38–40] and ligation of constitutively expressed Fas/CD95 on hepatocytes by antibodies results in rapid apoptosis [45–47].

Both bax and bcl-x proteins appeared to be upregulated in biliary epithelium in PBC cases. The expression of bax is induced by p53 [25,26] and the protein heterodimerizes with bcl-2, inhibiting the anti-apoptotic effect of bcl-2 [48]. We did not see upregulation of p53, suggesting p53-independent induction of bax and bcl-x may be occurring, as has been reported by others [49]. The bcl-x gene codes for two splice variants, full length bcl-x_l protein and a smaller bcl-x_s [50]. bcl-x_l protects cells from apoptosis and bcl-x_s blocks the protective effect of bcl-2 and bcl-x_l, acting as an 'anti-anti-apoptosis' protein. It is the relative ratio of homo- and heterodimeric forms of these interactive proteins which affects the apoptotic pathway. The polyclonal serum against bcl-x used does not discriminate between the long and short forms and hence we cannot draw any conclusions as to their relative ratios and role in regulating cell death in PBC biliary epithelial cells.

We have shown that the presence of proteins involved in executing apoptosis in biliary epithelial cells in PBC is consistent with previous reports of apoptosis of these cells [32,41,42]. We do not know what causes this apoptosis but we have also shown these cells are capable of regeneration. It may be that a change in the balance between apoptosis and regeneration of biliary epithelial cells during the course of disease contributes to the ultimate loss of bile ducts in PBC. However, because of the prolonged time course of this chronic disease and the secondary effects and immune response resulting from bile duct loss, intervention of apoptosis is unlikely to be a therapeutic target in the treatment of PBC.

Acknowledgements

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